

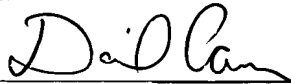
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**FUSION PROTEIN
COMPRISING TATDMT POLYPEPTIDE**

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FUSION PROTEIN COMPRISING TATDMT POLYPEPTIDE**BACKGROUND ART**

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent for acquired immunodeficiency syndrome (AIDS). The HIV-1 long terminal repeat (LTR) contains promoter elements responsible for the initiation of viral transcription. After synthesis, the full-length viral RNA is either transported directly into the cytoplasm and becomes translated into structural proteins or it is spliced into subgenomic RNAs that encode the several viral regulatory proteins. One of these regulatory proteins is the Tat protein, which is localized within the nucleus. Tat is essential for viral replication, and high level expression of HIV genome requires transcription by Tat.

The control of viral gene transcription is of prime importance in the viral replication. Control of transcription at the HIV-1 LTR promoter involves complex interactions among *cis*-acting elements, viral trans-activator, and cellular proteins. The key players of transcription regulation are Sp1, NF- κ B, Tat, P-TEFb, and TAR sequence. Basal transcription is contributed by Sp1 and TFIID. Tat drastically activates transcription either by increasing transcriptional initiation and/or elongation, and is essential for viral replication. Tat binds to the TAR sequence of the stalled short HIV-1 transcript, and interacts with Sp1, NF- κ B, TFIID and TFIIH. Tat also interacts with the cellular kinase (P-TEFb) and establishes the Tat-TAR-P-TEFb complex. Investigation on the transcription regulation of HIV-1 provides a molecular basis for developing novel antiviral agents. Developing therapeutic agents that can potentially repress transcription at the HIV-1 LTR promoter is important to overcome viral resistance, because such agents would prevent production of the genetic material for viral replication and the template for reverse transcriptase. Because Sp1 and Tat play a central role in the transcription, molecular tool that inhibit their activities are likely to repress transcription and replication of HIV-1.

A wide range of organism has transcription factors containing zinc-finger motifs, which bind to DNA in a sequence-specific manner. There are several types of zinc-fingers, but members of the Cys2-His2 class are ideal for generating artificial transcription factors owing to their diversity and modular structure. Unlike other DNA-binding domains, zinc-fingers are highly variable in terms of DNA sequence they recognize. Naturally occurring zinc-finger proteins show extremely diverse DNA-binding specificities, and recognize many different DNA sequences. Using

powerful design and selection methods, one can construct novel zinc-finger proteins that bind to almost any pre-determined DNA sequence of 9 to 20 nucleotides in length.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows that TatdMt fusions are targeted efficiently to the HIV-1 LTR and potentially repress transcription in stable HeLa cells. (A) Structures of TatWt and TatdMt fusion polypeptides. (B) Transient transfection assays of TatWt or TatWt fusions. The TatWt and TatWt fusion proteins, in the absence of TatWt, potentially activate transcription. (C) Transient transfection assays of TatdMt fusions in the presence or absence of the TatWt. □, transient transfection in the absence of TatWt. ■, assays in the presence of TatWt. (D) Titration of transcription inhibition by FBI-1-TatdMt fusion. FBI-1-TatdMt competed with TatWt and potentially inhibited transcription activation by TatWt. The average of three independent assays is shown. Bars (I) represent standard deviations.

Fig. 2 shows scheme of single-round HIV-1 virus replication inhibition assay transcription. HIV-1 proviral DNA, YK177-86, was cotransfected with an envelope protein VSV-G expression plasmid and the control pHYK plasmid or the effector FBI-1-TatdMt fusion plasmid into 293T cells. Cell-free medium was collected, diluted, and used to infect HeLaT4 cells or Magi cells. The numbers of infected cells were scored as described in Methods.

Fig. 3 shows that FBI-TatdMt potentially represses HIV-1 replication. (A) fresh medium was used for infection of HeLaT4 and Magi cells instead of virus supernatant. +/-, less than 100 blue cells per dish, ++++++, 15-20% of total cell population is stained blue. The results are averages of four independent experiments. (B) X-Gal staining of the Magi cells transfected with 1x or 100x diluent of HIV-1 viral supernatant produced from the 293T cells transfected either with pHYK or FBI-1-TatdMt expression plasmids. FBI-1-TatdMt drastically reduced the number of viral particle production. Compare 10^{-2} pHYK control with 10^{-2} FBI-TatdMt.

Fig. 4 shows that TatdMt fusion polypeptides do not alter the expressions of other cellular proteins in CV-1 cells (A). Total cell extracts (30 ng) were prepared from CV-1 cells transfected with TatWt, TatdMt, TatdMt fusion plasmids and analyzed by 10% SDS-PAGE and Western blot analysis. TatdMt fusion polypeptides do not induce apoptotic cell death. (B) Apoptotic cell death assay with transfected HeLa/Hyg^R cells. Total viable cells were counted by Trypan blue dye exclusion. To determine the viability of the transfected cells, cells expressing green fluorescence were counted

under a Zeiss fluorescence photomicroscope. Cells undergoing apoptosis were detected by DAPI staining. (C) Nuclear condensation and fragmentation were monitored by DAPI staining. Nuclear condensation and fragmentation were evident only in the cells transfected with TatWt.

5 Fig. 5 shows mapping of functionally important functional domains of FBI-1-TatdMT fusion. POZ-domain, TatdMt, and zinc-fingers are important domains in the repression of transcription activation by TatWt at HIV-1 LTR. (A) diagram of various fusion constructs. various deletions or mutations of parent construct FBI-1-TatdMt 1-1 were made. (B) Transcription inhibition assays of HIV-1 LTR by various fusion
10 constructs in stable HeLa cells. (C) comparison of ZF, 4-1ZFC and 1-1 in transcription repression. TatdMT fusion FBI-1 ZFs are targeted efficiently to the HIV-1 LTR and potentially repress transcription in. NLS(nuclear localization signal); ZF, zinc-finger; Tat, transactivator of transcription.

Fig. 6 shows design and sites of artificial zinc-finger proteins recognizing the
15 various regions of HIV-1 LTR. (A) Nucleotide sequence of the HIV-1 LTR and the regions where artificial zinc-fingers were designed to bind. Shaded bars are the regions where fingers were designed to bind. • indicates the upper most nucleotide of 9 or 12 bp recognition site of artificial zinc-finger. (B) Transient transcription inhibition assays of artificial zinc-fingers in stable HeLa cells with integrated HIV-1
20 LTR CAT gene. Artificial zinc-finger protein (AZF39) recognizing the HIV-1 LTR region -64 bp from transcription start site is the most potent transcription repressor in stable HeLa cells integrated with HIV-1 LTR.

Fig. 7 shows that POZ-domain, TatdMt fusion proteins of artificial zinc-finger 39 and 40 (AZF39, AZF40) recognizing the -64 bp ~ -56 bp and -67 bp ~ -59 bp sites of
25 HIV-1 LTR null HIV-1 transcription. (A) Diagram of FBI-1-TatdMt (1-1), artificial zinc-fingers AZF39, AZF40 and their fusions. (B) Transcription repression assays in stable HeLa cells.

Fig. 8 shows that POZ-domain, TatdMt fusion proteins of artificial zinc-finger potentially inhibit HIV-1 replication, as revealed by transient trans complementation
30 virus replication inhibition assays. (A) microscopic pictures of X-gal stained Maggi cells infected with recombinant HIV-1 virus at 50-100x magnification. (B) Table of replication inhibition by various fusion proteins.

DISCLOSURE OF INVENTION

35 The present invention provides novel anti-AIDS therapeutic agents related with TatdMt fusion proteins.

According to the present invention, inventors provided the fusion proteins comprising artificial zinc-fingers, which recognize human immunodeficiency virus (HIV) long terminal repeat (LTR) region and contain TatdMt polypeptide.

5 The fusion proteins of the present invention further comprises POZ (Poxvirus zinc finger)-domain.

The fusion proteins of the present invention can further comprise a nuclear localization signal (NLS).

10 The fusion proteins of the present invention recognize the HIV-1 LTR region region -64 bp ~ -56 bp site (AZF39) and/or -67 bp ~ -59 bp site (AZF40) from transcription start site, respectively.

The zinc-finger in the present invention can comprise amino acid sequence of SEQ ID NO:11 (AZF39) or SEQ ID NO:12(AZF40).

The TatdMt polypeptide in the present invention can comprise 73amino acid of SEQ ID NO:10.

15 The POZ-domain in the present invention can comprise amino acid sequence of SEQ ID NO:9.

The fusion proteins of the present invention are suitable for use as anti-AIDS therapeutic agents.

20 According to the present invention, there is provided a DNA molecule that encodes a fusion protein comprising a zinc finger and a TatdMt polypeptide.

The zinc finger in the present invention can comprise nucleic acid sequence of SEQ ID NO:13 (AZF39) or SEQ ID NO:14(AZF40).

The TatdMt polypeptide in the present invention can comprise nucleic acid sequence of SEQ ID NO:15.

25 According to the present invention, there is provided a DNA molecule that encodes a fusion protein comprising a POZ (Poxvirus and Zinc finger)-domain; a zinc finger; and a TatdMt polypeptide.

The POZ-domain in the present invention can comprise nucleic acid sequence of SEQ ID NO:16.

30 Inventors designed and prepared novel artificial zinc-finger proteins and their fusion proteins that can recognize the HIV-1 LTR promoter sequence. Inventors tested the transcription repression activities of the zinc-fingers and found that they can repress transcription of HIV-1 LTR by Tat potently, particularly when the fingers were fused with transcription repression domain POZ-domain and Tat mutant form
35 lacking its ability to interact with PTEFb.

Investigation on the transcription regulation of HIV-1 provides a molecular basis

for developing novel anti-viral agents. Developing therapeutic agents that can potentially repress transcription at the HIV-1 LTR promoter is important to overcome viral resistance, because such agents would prevent production of the genetic material for viral replication and the template for reverse transcriptase. Inventors
5 designed and prepared the fusion gene constructs that express the POZ-domain of FBI-1, full-length FBI-1 or artificial zinc-finger polypeptides fused to a particular mutant version of Tat (TatdMt) and investigated their functions in the repression of HIV-1 transcription and replication. The fusions were efficiently targeted to the HIV-1 LTR. The fusions potentially blocked transcription activation by Sp1 and Tat and, in
10 particular, FBI-1-TatdMt fusion completely blocked transcription and repressed HIV-1 replication by as much as 230 fold.

Based on this discovery, we investigated the domains of FBI-1-TatdMt important in transcription repression of HIV-1 and found that POZ-domain, zinc-finger DNA binding domain, and TatdMt are important. Because FBI-1 is a ubiquitously
15 transcription factors and can potentially affect other gene expression once it was introduced into target cells, we prepared artificial zinc fingers that recognize the HIV-1 LTR specifically to circumvent this potentially problem. The artificial zinc-finger polypeptides fused with POZ-domain and TatdMt were targeted to the Sp1 binding site (-64 to -56 bp, -67 to -59 bp upstream of transcriptions start site) and the
20 completely blocked transcription activation by Sp1 and Tat.

The following embodiments and results illustrate the invention.

EMBODIMENTS

Materials and Methods

25

Plasmid Constructs and Antibodies.

The HIV-1 LTR-CAT fusion plasmid (pU3R-IIICAT) has been described previously (Sodroski JG et al. (1985) Science 227, 171-173). The expression plasmids for TatWt (HIVHBX2R type, 86 a.a.), TatdMt (Tat^{K28A,K50A}, 72 a.a.), and
30 FBI-1 were prepared by subcloning their cDNAs into pcDNA3.0 (Invitrogen) (Kiernan RE, et al., (1999) EMBO J. 18, 6106-6118; Karn, J. (1999) J. Mol. Biol. 29, 235-254; Lee DK et al. , (2002) J. Biol. Chem. 277, 26761-26768). The expression plasmids for the POZ-domain fusion proteins either with TatWt or mutant TatdMt were constructed by subcloning their cDNAs into pcDNA3.0-TatWt or -TatdMt
35 plasmids.

Various deletion and zinc-finger mutants of FBI-1 were prepared by subcloning

PCRed FBI-1 or FBI-1 zinc-finger 1 mutant cDNA fragments into pcDNA3.0-TatdMt plasmid (Fig. 1A) (Lee, D.-K., et al., (2002) J. Biol. Chem. 277, 26761-26768; Doetzlhofer, A., et al., (1999) Mol. Cell. Biol. 19, 5504-5511; Bartz, S. R., et al., (1999) J. Virol. 73, 1956-1963). For PCR reaction to amplify the region between a.a. 124 to a.a. 584 for constructs 2-1 and 2-2 (figure 5A), forward 5'-ACGTAAGCTTACCATGGCGCCGACCTCCTGGACCGG-3' (primer corresponding to a.a. 124 from N-terminus; SEQ ID NO:1) and, reverse 5'-GATCGAATTCGGCGAGTCCGGCTGTGAAGTT-3' (primer corresponding to a.a. 584 from N-terminus; SEQ ID NO:2) were used. To amplify the region between a.a. 124 to a.a. 512 for constructs 3-1 and 3-2, the forward 5'-ACGTAAGCTTACCATGGCGCCGACCTCCTGGACCGG-3'(SEQ ID NO:1) and reverse 5'- GATCGAATTCGGGGCTGGGGTCGGGCGCCCCGCC-3'(primer corresponding to a.a. 512 from N-terminus; SEQ ID NO:1) were used. Also for PCR reaction between a.a. 335 and a.a. 584 (for 4-1 construct) or a.a. 512 (for 5-1, 5-2 constructs), forward 5'-ACGTAAGCTTACCATGGGGGACAGCGACGAGTC-3' (primer corresponding to a.a. 335 from N-terminus; SEQ ID NO:4) was paired either with reverse primer(SEQ ID NO:5) 5'-GATCGAATTCGGCGAGTCCGGCTGTGAAGTT-3' or 5'-GATCGAATTCCGGGGCTGGGGTCGGGCGCCCCGCC-3' (SEQ ID NO:3). For 4-1ZFC, forward 5'-ACGTAAGCTTACCATGGGGGACAGCGACGAGTC-3' (SEQ ID NO:6) primer was paired with reverse primer 5'-GATCGAATTCGGCGAGTCCGGCTGTGAAGTT-3' (SEQ ID NO:5) primer for PCR reaction. To PCR ZF only region, forward primer 5'-ACGTAAGCTTACCATGGAGAAGGTGGAGAAGATCCGA-3' (primer corresponding to a.a. 371 from N-terminus; SEQ ID NO:7) and reverse primer 5'-ACGTAAGCTTCGAGGGGACGCCGTTGCAGCC-3' (primer corresponding to a.a. 495 from N-terminus; SEQ ID NO:8) were used.

PCR was performed by denaturing at 94°C for 5 min, 30 cycles of amplification reaction (94°C 30 sec, 60°C 1 min, 72°C 3 min), and final extension at 72°C for 4 min. For PCR reactions to prepare 1-1, 2-1, 3-1, 4-1, 5-1, 4-1ZFC, and ZF fragment, Inventors used the FBI-1 cDNA that Inventors isolated previously (full length FBI-1 cDNA cloned into pBluscriptI(KS-) as PCR template (Doetzlhofer, A., et al., (1999) Mol. Cell. Biol. 19, 5504-5511). For PCR reactions of 2-2, 3-2, and 5-2, Inventors used the FBI-1 cDNA with mutation at the first zinc-finger (a.a. C384A, C387A), which was kindly provided by Dr. Nuria Hernandez of Cold Spring Harbour Laboratory (New York, USA).

The amplified PCR products were cloned into TOPO TA cloning vector (pCR[®]2.1-TOPO[®], Invitrogen). The resulting ligated mixtures were transformed into competent *E. coli* DH5 α and the transformed *E. coli* were grown on LB-agar-Ampicillin-X-gal-IPTG selection plates. White *E. coli* colonies were grown in LB-Ampicillin broth and plasmids were prepared at 1.5 ml mini-scale. The plasmids with PCR products inserts were digested with *Hind*III and *Eco*RI by standard alkaline lysis protocol, inserts were purified by agarose gel electrophoresis and cloned into pcDNA3.0TatdMt plasmid to generate various deletion mutant of FBI-1-TatdMt. PCRRed ZF cDNA of FBI-1 was prepared in the same manner and cloned into pcDNA3.0 digested with *Hind*III.

Ten artificial zinc-fingers cloned in pcDNA3.1 (Invitrogen, CA, USA) were designed and prepared by ToolGen Inc.(Taejeon, Korea). The fingers were designed to recognize the regions such as Sp1 binding site, TATA box, and FBI-1 binding inducer of short transcript sequence that are critical in transcription of HIV-1 LTR. They usually contain either 3 zinc-fingers (clone -71, -68, -67, -64, -61, -58-I, -55, -28, +41; the numbers are the upper-most nucleotide of the recognition sites counted from the transcription start site on the HIV-1 LTR) or 4 zinc-fingers (-58-II), which recognize 9 bp or 12 bp sequence of the HIV-1 LTR. All the artificial zinc-fingers contain the same 5' and 3' sequences flanking the zinc-finger open reading frame (ORF) sequences. To prepare, the pcDNA3.0-artificial zinc-finger fusion TatdMt constructs, forward 5'-GATCGGTACCA

TGGAATTGCCTCCAAAAAGAAG-3'(SEQ ID NO:21) and, reverse 5'-GATCGATATCTGCGGCCGCTTTTTCACCGGTATG-3' (SEQ ID NO:22) were used to PCR out the zinc-finger open reading frame (ORF) sequences using various artificial zinc-finger plasmids as PCR templates. PCR was performed by denaturing at 94°C for 5 min, 30 cycles of amplification reaction (94°C 30 sec, 60°C 1 min, 72°C 3 min), and final extension at 72°C for 4 min. The PCR products were digested with *Hind*III and *Eco*RV and cloned into pcDNA3.0-TatdMt plasmid digested with *Hind*III-*Eco*RV.

The antibodies against various proteins such as actin, tubulin, p21^{Waf/Cip1}, Sp1, AKT, ERK, GSK, mSin3A are from either Santa Cruz Biotechnology or Upstate Biotechnology (CA, USA).

Cell culture and transient transfection reporter assays.

The stable HeLa cells intergrated with HIV-1-LTR-CAT gene fusion were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal

bovine serum and penicillin (100 units/ml)/streptomycin (100 µg/ml) (Invitrogen, CA, USA).

Cells were inoculated on a six-well tissue culture plates at a density of 1×10^5 cells/well in 2 ml of DMEM medium. After growing the cells for 24 hrs, cells were transiently transfected with mixture of plasmids pcDNA3.0 TatWt expression plasmid (0.3µg), pCMV-*LacZ* plasmid (0.1 µg), and various pcDNA3.0 expression plasmids (0.3 µg) encoding TatWt, TatdMt, TatWt or TatdMt fused either with FBI-1 or POZ-domain using Lipofectamine Plus reagent (Invitrogen, CA, USA) according to the manufacturer's recommended protocol (Fig. 1). For titration experiments, 0-0.3 µg of pcDNA3.0-FBI-1-TatdMt was cotransfected with pcDNA3.0 TatWt (0.3 µg), and pCMV-*LacZ* plasmid (0.1 µg) (Fig. 1D). Cells were harvested and lysed in 150 µl of reporter lysis buffer and vortexed for 1 min and centrifuged at 1,2000 rpm, for 3 min at 4 °C. Cellular extracts (20 µl) were analyzed for β-galactosidase activity by mixing with 180 µl of substrate solution (o-nitrophenyl-β-D-galactopyranoside 4 mg/ml in 0.1 M sodium phosphate, pH7.5; 0.1 M sodium phosphate buffer, pH 7.5; 100× Mg²⁺ (0.1 M MgCl₂; 4.5 M β-mercaptoethanol).

For reporter CAT (chloramphenicol acetyltransferase) gene expression assays, cells were grown for 24 hrs after transfection. Cells are washed three times with PBS and collected the cells after addition of 1ml TEN buffer to each well. The harvested cell pellet was resuspended 75 or 150 µl of 0.25 M Tris-HCl, pH 7.8. Cells are lysed by three repeated freeze-and-thaw cycles using EtOH/ dry ice bath. Variations in transfection efficiencies were normalized using co-expressed β-galactosidase activity. Each CAT reaction assay mixtures contains 50 µl 0.25 M Tris-HCl , pH 7.8, 10 µl 4mM Acetyl CoA (3.5 mg/ml), 25 µl cell extract, 0.5 µl ¹⁴C-Chloramphenicol (CAM) (25nCi/ µl) and incubated for 15 min at 37 °C. Reactions are stopped by adding 1ml of ethyl acetate and dried under vacuum using the Speed-Vac concentrator. The Ac-CAM and CAM were separated by Silica gel TLC (Aldrich, MO, USA) using CHCl₃:Methanol=95:5 as developing solvent system in a TLC jar. Dried TLC plate was exposed to Fuji Phosphor Image plate and analyzed by Fuji phosphor image analyzer (Tokyo, Japan).

HIV-1 replication inhibition analysis-Transient trans-complementation.

HIV-1 replication inhibition analysis was carried out as described elsewhere(Kim YS, et al., (1996) J. Neurosci. Res. 43, 652-663; Park IW, et al., (2001) J. Immunol. 167, 2766-2771.; Kimpton, J., et al., (1992) J. Virol. 66, 2232-2239.; Selliah N, et al., (2001) Cell Death Differ. 8, 127-136.). A plasmid that expresses HIV-1 genome with

the Hygromycin B resistance gene was cotransfected into 293T cells with a viral envelope protein VSV-G expression plasmid and either the control (pHYK) or the TatdMt fusion protein expression plasmid. The cotransfection resulted in the production of HIV-1 virus particles that undergo only a single round of infection in susceptible cell lines such as HeLaT4 and Magi cells (Kim YS, et al., (1996) J. Neurosci. Res. 43, 652-663; Selliah N, et al., (2001) Cell Death Differ. 8, 127-136). The efficiency of a single round of infection was determined by scoring the numbers of *Hyg*^R HeLaT4 cells or β -galactosidase positive Magi cells (Fig. 2A). To produce HIV-1 virus, 293T cells were transfected with HIV-1 proviral DNA (YK177-86, 1 μ g), either control (pHYK, 2 μ g) or FBI-1-TatdMt fusion expression plasmid (2 μ g), envelope protein expression plasmid (pVSV-G, 0.5 μ g), and *LacZ* gene expression plasmid (MFG/*LacZ*/Puro, 0.5 μ g) using Lipofectamine plus reagent (Gibco-BRL). MFG/*LacZ*/Puro plasmid was cotransfected to normalize variations in transfection efficiencies. The transfected cells were incubated further for two days to prepare supernatant containing infectious HIV-1 virus particles. The supernatant from the transfected 293T cells was collected, filtered, diluted and used to infect CD4⁺ HeLaT4 cells and Magi cells (HeLaT4 cells integrated with HIV-1 LTR-*LacZ* gene). After 4 hrs, fresh culture medium was added and cells were cultured for two days. The virus titer was determined by scoring the number of Magi cells that turned blue after β -galactosidase staining. In the case of HeLaT4 cells, *Hyg*^R positive cells were selected with Hygromycin, cultured for another 10 days, and the virus titer was determined by counting colonies that were fixed and stained with 0.5% crystal violet/50% (v/v) methanol. Assays results are presented as averages of four independent experiments.

Western blot analysis of cellular proteins.

To investigate whether fusion proteins change the expression of cellular proteins in transfected cells, the expressions of constitutively expressed genes (actin, tubulin, TCTP p21), components of signal transduction (Akt, Erk, and GSK), transcription factor (Sp1), and co-repressor (mSin3A) were examined. Total cell extracts were prepared from the CV-1 cells transiently transfected with a mixture of HIV-1 LTR luciferase plasmid (0.6 μ g), TatWt (0.3 μ g) or various TatdMt fusion expression plasmids (0.3 μ g) (Fig. 1A). Proteins (30 μ g) were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and analyzed by Western blotting using various antibodies and an ECL-detection kit (Amersham).

Cell viability and apoptosis.

Hygromycin resistant HeLa (HeLa/Hyg^R) cell line stably established by transfection with a Hygromycin B phosphotransferase gene expression vector was maintained in DMEM with 10% fetal calf serum. The cell line is useful in assessing the apoptotic activity of Tat polypeptides because Tat induces the apoptosis of cell without sensitization, such as serum starvation or TNF- α treatment, as was observed in microvascular endothelial cells(Bartz, S. R., et al., (1999) J. Virol. 73, 1956-1963.; Cullen BR. (1993) Cell 373-370.). Cells were transfected with TatWt or TatdMt fusion expression plasmid (0.5 μ g) and GFP expression plasmid (0.5 μ g) using Fugene 6 (Roche, NJ, USA). 24 hours after transfection, total viable cells were counted by Trypan blue dye exclusion. To determine the viability of transfected cells, cells with green fluorescence were counted using a fluorescent photomicroscope (Zeiss, Germany). Cells undergoing apoptosis were detected by DAPI staining 21 hours after transfection.

Design and preparation of artificial zinc-fingers recognizing the HIV-1 LTR.

The Artificial zinc-fingers recognizing the HIV-1 LTR were designed to contain 3 or 4 zinc-fingers and accordingly recognize 9 bp or 12 bp nucleotide sequence of the HIV-1 LTR. The targeted site of the zinc fingers are Sp1 binding region (-78 bp to -45 bp), TATA box region (-37 bp to -3 bp), and inducer of short transcript region (+37 bp to +69 bp). They are named as clone -71, -68, -67, -64, -61, -58-I, -58-II, -55, -28, and +41. The numbers indicate the upper-most nucleotide of the recognition sites counted from the transcription start site on the HIV-1 LTR. Except -58-II which contains 4 zinc-fingers, all the zinc-fingers contain 3 zinc-fingers.

Transcription inhibition assays of the artificial zinc-fingers or artificial zinc-fingers fused with POZ-domain and TatdMt, and transient transfection reporter CAT assays.

The stable HeLa cells integrated with HIV-1 LTR chloramphenicol acetyl transferase (CAT) gene was grown in Dubelco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 μ g/ml) (Invitrogen, CA, USA). Plasmids used prepared a by alkaline lysis methods in large scale (250 ml) and purified by CsCl/EtBr ultra centrifugation. Cells were inoculated on a six-well tissue culture plates at a density of 1×10^5 cells/well in 2 ml of DMEM medium. After growing the cells for 24 hrs, cells were transiently transfected with 0.3 μ g of each pcDNA 3.1 zinc-finger expression

plasmid, 0.3 µg of pcDNA3.0-TatWt expression plasmid, and 0.1 µg of pCMV-β-galactosidase (LacZ) mixed with LipofectAmine Plus reagent (Invitrogen) according to the manufacturer's recommended protocol in serum free DMEM medium. After 3 hrs, cells were supplied with fresh complete DMEM and allowed to grow for 24 hrs.

5 Rest of the assay procedures are the same as described above in the transient transfection reporter assays in the Materials and Methods. Assays were repeated 3 times.

To investigate the transcription inhibition properties of the artificial zinc-fingers fused with POZ-domain and TatdMt, the stable HeLa cells were transfected with

10 plasmid mixture composed of 0.3 µg of pcDNA3.0 artificial zinc-finger-TatdMt, 0.3 µg pcDNA3.0-TatdMt, and 0.1 µg pCMV-β-galactosidase (*LacZ*) using Lipopectamine plus reagent (Invitrogen, CA, USA). Rest of the transcription assay procedures are the same as described above.

15 Results

1. TatdMt fusions are targeted efficiently to HIV-1 LTR.

In order to develop of novel anti-AIDS therapeutic agents that can repress HIV-1 gene transcription in vivo by disrupting the interaction of the two major activators, inventors fused the Sp1 inhibiting polypeptides (FBI-1 POZ domain, FBI-1) to either

20 the N or C terminus of TatWt polypeptide (86 a.a.)(Fig. 1A). The TatWt fusion expression plasmids were transfected into the stable HeLa cells with integrated HIV-1 LTR-CAT fusion gene. In the absence of TatWt, transcription occurred at a low basal level (Fig. 1B, lane 1). TatWt potently activated transcription by 55 fold (Fig.

25 1B, lane 2). Unexpectedly, the Sp1 inhibitory polypeptides did not show any repression and the four TatWt fusions potently activated transcription instead (Fig. 1B, lanes 3-6). In particular, FBI-1-TatWt activated transcription more potently than TatWt alone, probably reminiscent of cooperative interaction between Tat and FBI-1 in this particular configuration. Although the result was rather unexpected and

30 disappointing, the results clearly suggest that the fusion proteins were targeted efficiently to HIV-1 LTR and have full potential to activate transcription after they bind to TAR. The ability of TatWt to interact with P-TEFb and activate transcription appears to mask the inhibitory potential of fused Sp1 inhibitory polypeptide on transcription. Also, the fusion proteins were unable to repress transcription activation

35 by TatWt .

For targeting purposes, inventors adopted a different version of Tat

(TatdMt^{K28A&K50A}) with no ability to interact with P-TEFb, but with a two fold stronger interaction with TAR than TatWt (Kiernan RE, et al., (1999) EMBO J. 18, 6106-6118). The two lysine residues of Tat at a.a. 28 and 50 that are critical for interaction with cyclin T1, were replaced with alanines in the Tat mutant.

5 Accordingly, the TatdMt neither formed a complex with P-TEFb (cyclin T1 and CDK9) nor activated transcription (Figure 1C, white column, lane 2). The features of TatdMt are ideal for targeting the Sp1 inhibiting polypeptides to the HIV-1 LTR because the fusion protein can be delivered more efficiently to TAR and stays longer and, even more importantly, there will be no phosphorylation of the CTD of RNA

10 polymerase II. Inventors prepared the Sp1 inhibiting proteins fused with the TatdMt in two possible configurations (Figure 1A bottom: POZ-TatdMt and FBI-1-TatdMt, only structures in one configuration are shown; Figure 2A). The fusion expression plasmids were transfected into the stable HeLa cells or CV-1 cells in the presence or absence of TatWt expression plasmid, and analyzed for reporter CAT or luciferase

15 activity. TatWt on its own potently activated transcription, by more than 46 fold (Figure 1C filled bar, lane 1). In contrast, TatdMt and the TatdMt fusion proteins alone did not activate transcription over the basal level (Figure 1C open bars, compare lanes 1 vs. 2-4). However, TatdMt reduced transcription activation by TatWt to half of that observed with TatWt alone, suggesting that there is competition for the

20 TAR sequence between the two (Figure 1C, compare filled bars, lanes 1, 2). The TatdMt fusion proteins not only successfully competed with TatWt for TAR, but also repressed transcription potently even in the presence of excess TatWt expression plasmid (0.3 µg) (Figure 1C, compare filled bars, lanes 1 vs. 3, 4). The fusion proteins repressed transcription in either orientations (data not shown). FBI-1-TatdMt

25 was the most potent repressor among the tested fusion proteins (TatdMt fusion with MeCP2, HDAC1) and repressed transcription down to basal level (data not shown). The POZ-domain (1-123 a.a.) of FBI-1 also showed potent repressor activity, which suggests that the domain is responsible for the repressor activity of FBI-1-TatdMt fusion protein (Fig. 1C, filled bar, lane 3). Inventors further titrated the transcription

30 repression by FBI-1-TatdMt (Fig. 1D). FBI-1-TatdMt competed with TatWt and potently inhibited transcription by more than 30% at 3 ng, 80% at 30 ng, and abolished transcription at 300 ng (Fig 1D, lanes 3-5).

2. Transient trans-complementation assays or single-round HIV-1 virus replication inhibition assay.

35 Inventors investigated the effect of the TatdMt fusion proteins on the HIV-1 replication using transient trans-complementation assays in 293T cells. Figure 2

shows the scheme of single-round HIV-1 virus replication inhibition assay. HIV-1 proviral DNA, YK177-86, was cotransfected with an envelope protein VSV-G expression plasmid and the control pHYK plasmid or the effector FBI-1-TatdMt fusion plasmid into 293T cells. HIV-1 proviral DNA, YK177-86, do not have a gene
5 encoding an envelope protein that is essential in the packing of HIV-1 virus. By coexpressing the envelope protein in 293T cells, infectious HIV-1 virus can be produced. The resulting virus cannot replicate in the infected cells because neither host cell nor the HIV-1 YK177-86 proviral DNA cannot provide envelope protein. By co-expressing the FBI-1 TatdMt fusion protein in the 293T cells, the transcription at
10 the HIV-1 can be modulated, which in turn translated into the change in the number of infectious HIV-1 virus.

After cells are transfected with the plasmid mixtures, cell-free medium was collected, diluted, and used to infect host cells such as HeLaT4 cells or Magi cells. The number of infectious HIV-1 particles produced were counted by transfection and
15 Hyg selection or X-gal staining of HeLaT4 or Magi cells. X-gal staining of the Magi cells infected with 100 fold diluents of HIV-1 viral supernatants from the 293T cells transfected with FBI-TatdMt expression plasmid, showed that a few viral infection was detected in contrast to pHYK control which showed several hundreds viral transfected cells.

20 This suggested that FBI-TatdMt repressed transcription of HIV-1 and reduced the number of infectious HIV-1 virus particles produced from 293T cells by as much as 100-230 fold (580 ± 80 vs. 6 ± 1 at 10x dilution; 5800 ± 800 vs. 25 ± 1 at 1x dilution) compared to negative control PHYK (Fig. 3).

25 **3. The specificity of TatdMt fusion proteins.**

To be useful as therapeutic agent against AIDS, the fusion proteins should act specifically at the HIV-1 LTR promoter, and ideally, they should have little effect on the expressions of cellular genes. Inventors investigated whether over expression of the TatdMt fusion proteins change the expression of various cellular proteins in
30 transfected CV-1 cells by Western blot analysis (Fig. 4A). The expression of constitutively expressed genes (actin, tubulin, TCTP p21), component of signal transduction (Akt, Erk, GSK), transcription factor (Sp1), and co-repressor (mSin3A) was not altered by the expression of various Tat or fusion proteins, suggesting that the fusions act specifically at the HIV-1 LTR (Fig. 4A).

35

4. Apoptotic cell death assay with transfected HeLa/Hyg^R cells.

Tat sensitizes and induces apoptosis in HIV-1 infected or Tat-transfected cells. Inventors tested whether the TatdMt fusion polypeptides have apoptotic activity in Hyg^R HeLa clonal cells (HeLa/Hyg^R). HeLa/Hyg^R cells were cotransfected with TatWt (or fusion polypeptides with TatdMt) and green fluorescence protein (GFP) expression plasmids, and analyzed for apoptosis after 21 hrs. Cells were analyzed both for GFP expression and for apoptosis by viable cell counting using trypan blue dye exclusion and DAPI staining. Transfection with TatWt expression plasmid resulted in a significant decrease in the number of viable cells and about 80% of the cells transfected with TatWt underwent apoptosis (Fig. 4B, lane 2). However, the cells transfected with TatdMt showed much weaker apoptotic activity and only 10% of the transfected cells underwent cell death (Fig. 4B, lane 3). The cells transfected with TatdMt fusion polypeptides did not show any sign of cell death and were identical to mock transfected cells (Fig. 4B, compare lane 1 vs. 4, 5). Inventors also investigated the effects of TatWt or TatdMt-fusion polypeptides on nuclear condensation. Twenty-one hours after transfection, HeLa/Hyg^R cells were fixed and stained with DAPI. Once, the cells were transfected with TatWt expression plasmid, the total number of viable cells significantly decreased. TatWt induced nuclear condensation and, in contrast, FBI-1-TatdMt fusion proteins did not (Fig. 4B).

5. Mapping of functionally important functional domains of FBI-1-TatdMT fusion.

Inventors investigated which domains of FBI-1-TatMt contribute in the potent transcription repression by the FBI-1-TatdMt. Inventors prepared deletion or mutant constructs (Fig. 5A). Deletion was made with the POZ-domain (1-123 a.a.), C-terminus (513-584 a.a.), and junctional domain between the POZ-domain and the zinc-fingers (124-334 a.a.). Also, to investigate importance of the zinc-finger DNA binding domain, mutations at the two cysteine residues of the first zinc-finger (C384A; C387A) were introduced. Transient transfection assays in the stable HeLa cells showed that the FBI-TatdMt again potently represses transcription activation by TatWt (Fig. 5B, compare lanes 2, 3). Deletion of POZ-domain (2-1) resulted in 5-fold decrease in transcription repression, suggesting the importance of the POZ-domain in repression by inhibiting Sp1 (Fig. 5B, compare lanes 3, 4). The inter domain region (124-334 a.a.) gave rather ambiguous result. In the constructs (2-1, 4-1) that retain C-terminus end, deletion of the inter domain region gave stronger repression. However, in the constructs with C-terminus removed (compare 3-1 vs 5-1), the deletion of the region showed weaker repression. Deletion of C-terminus (513-584 a.a.) also showed

ambiguous result on the repression. Upon comparison of 2-1 and 3-1, the C-terminus region might be not so important in repression. However, the data on 4-1 and 5-1 suggested that the C-terminus region is important, and 4-1 showed 7 fold more potent repression than 5-1.

5 All of the zinc-finger mutants (2-2, 3-2, 5-2) did not show repressor activity comparable to any of the deletion mutants and showed repression activity only comparable to TatdMt alone (Fig. 5C filled bar, lane 2). This suggested that the zinc finger is essential in transcription repression of the fusion constructs, probably by blocking progression of transcription machinery and also by helping the TatdMt in
10 TAR binding. Our data indicated that the POZ-domain and the zinc-finger are important repression domains of FBI-1-TatdMt. Intriguingly, inventors found that zinc-finger itself (ZF) with no nuclear localization signal (NLS) attached is targeted to nucleus and effectively repressed transcription, although relatively weak compared either to 4-1ZFC or FBI-1-TatdMt. FBI-1-TatdMt repressed transcription 5 times
15 stronger compared to zinc-finger alone (Fig. 5C compare lane 2 vs. 3, 4). Our data also suggest that although the zinc-finger of FBI-1 can repress transcription, the degree of transcription repression can be enhanced by being fused with TatdMt and POZ-domain (Figure 5C).

20 **6. Design and transcription repression by artificial zinc-fingers recognizing various regions of HIV-1 LTR.**

TatdMt fusion proteins were shown to repress transcription potently and, although limited in context, also shown to act specifically at that HIV-1 LTR. Inventors are still concerned with the specificity of the fusion proteins because FBI-1
25 is ubiquitously expressed and over expression of the fusion protein in the targeted cells can potentially affect or alter expression of various genes. Concerns on the specificity of the zinc-finger can be overcome by adopting the artificial zinc-fingers that recognize the HIV-1 LTR only.

Inventors designed and prepared 10 zinc-finger proteins (Fig. 6A). The artificial
30 zinc-fingers recognizing the HIV-1 LTR were designed to contain 3 or 4 zinc-fingers and accordingly recognize 9 bp or 12 bp nucleotide sequence of the HIV-1 LTR promoter. The targeted site of the zinc fingers are Sp1 binding region (-78 bp to -45 bp), TATA box region (-37 bp to -3 bp), and inducer of short transcript region (+37 bp to +69 bp) (Figure 6A). They are named as zinc-finger clone -71, -68, -67, -64, -
35 61, -58-I, -58-II, -55, -28, and +41. The numbers indicate the upper-most nucleotide of the recognition sites counted from the transcription start site on the HIV-1 LTR.

Except -58-II which contains 4 zinc-fingers, all the zinc-fingers contain 3 zinc-fingers.

The zinc-finger expression plasmids (0.3 µg) were cotransfected with TatWt expression plasmid (0.3 µg) into stable HeLa cells. Most of the artificial zinc-fingers do not repressed transcription significantly. Zinc-finger -58-II, -64, and -67 zinc-finger repressed transcription. In particular, artificial zinc-finger -64 repressed most potently among the tested fingers, but it did not repress transcription down to the basal level (Fig. 6B). Interestingly, the site is the recognition site by Sp1 transcription factor with highest affinity, one of the key regulators of HIV-1 transcription.

7. POZ-domain, TatdMt fusion proteins of artificial zinc-fingers recognizing the -64 to -56 bp and -67 to -59 bp Sp1 binding sites, potently repress transcription and HIV-1 replication.

In figure 5, inventors found that the functional domains important in transcriptional repression by FBI-1-TatdMt are POZ-domain, zinc-fingers, and TatdMt. And figure 6 showed that although the artificial zinc-finger can repress transcription, it alone cannot repress transcription to the basal level. Therefore, inventors fused the POZ-domain and TatdMt to the artificial zinc-fingers (-67 bp AZF40 and -64 bp AZF30 zinc-fingers) and tested their activities in transcription repression (figure 7).

Again, the zinc-fingers alone can only repress transcription down to 1/5-1/10 of the transcription level activated by TatWt. By being fused with TatdMt, the repressor activity is enhanced by 2-3 fold. The repressor activities of the AZF39 (-64 zinc-finger) or AZF40 (-67 zinc-finger) are further enhanced by being fused with POZ-domain and completely null transcription activation by TatWt (figure 7B).

The full amino acid sequences of AZF39 or AZF40 and TatdMt fusion proteins are represented in SEQ ID NO:17(AZF39-TatdMt) or SEQ ID NO:18(AZF40-TatdMt).

The full amino acid sequence of POZ-domain; AZF39 or AZF40; and TatdMt fusion proteins are represented in SEQ ID NO:19(POZ-domain -AZF39-TatdMt) or SEQ ID NO:20(POZ-domain -AZF40-TatdMt).

Inventors have so far shown that the POZ-domain, zinc-fingers and TatdMt of FBI-1-TatdMt fusion transcription repressor protein are important in inhibition of HIV-1 transcription and replication (Fig. 7). Others have shown that Sp1 binding GC-box site and Sp1 is important in the Cyclin T1 dependent transcription activation of

HIV-1 LTR. Artificial zinc-fingers that were designed to be targeted the Sp1 binding sites (-44 to -76bp) repressed transcription effectively. To potentiate and to confer specificity to the fusion protein to HIV-1 LTR, inventors fused the POZ-domain of FBI-1 and TatdMt to the artificial zinc fingers (AZF39, AZF40) and cloned the fusions in pcDNA 3.0 mammalian expression plasmid.

Inventors performed single round viral replication inhibition assays using Maggi cells as described in Materials and Methods and fig. 2. The single round transfection competent HIV-1 virus were produced by cotransfection of recombinant HIV-1 genome defective in envelope protein gene, VSV-G envelope protein gene, and effector expression plasmids (fusion transcription repressor) or control vector plasmid (pYHK) into virus packaging 293T cells. The viral supernatant was diluted and used to transfect the Maggi cells and, to count the number of cells transfected with HIV-1 virus, cells were stained with X-gal. The cells were viewed at two magnification 100X and 50X. Although FBI-1-TatdMt potently inhibited HIV-1 replication, the POZ-domain-AZF39-TatdMt and POZ-domain-AZF40-TatdMt inhibited replication more potently (fig. 8A). When the same volume of viral supernatant was used to transfect the cells, in the control experiment, about half of the Maggi cells were stained blue. However, in the cells transfected with POZ-AZF39-TatdMt or POZ-AZF40-TatdMt, only 2.6% or 0.6% of the total cells were stained blue. Our data suggest that newly AZF fusion proteins are much more effective replication inhibitors compare to FBI-1-TatdMt, approximately 5.5-21 fold. Our data imply that once the fusion expression plasmids are delivered to HIV-1 infected cells and expressed effectively, the fusion protein may potently inhibit HIV-1 virus transcription and replication.

INDUSTRIAL APPLICABILITY

As known from the above Examples and Results, by attaching the zinc-fingers to the Sp1 repression domain or chromatin compacting domain (e.g., POZ-domain) and TatdMt, the effectiveness of the zinc-fingers on transcription repression can be greatly increased. The zinc-finger of the FBI-1 or artificial zinc finger alone repressed transcription but at much lower efficiency. By being attached to both the POZ-domain and the TatdMt, the zinc finger fusion proteins become potent repressor and null the transcription activation of HIV-1 by the TatWt.

For treatment of AIDS, anti-HIV drugs and vaccines must overcome the viral acquisition of drug resistance caused by the high rate of mutation introduced during reverse transcription. One of the ways to overcome the viral resistance is to develop a

therapeutic agent that can block *de novo* synthesis of viral RNA transcripts from the proviral HIV-1 genome. The FBI-1-TatdMt fusion, the artificial zinc-finger-POZ-domain-TatdMt fusion proteins, and molecular approach developed here provide an effective therapeutic agent or strategy against AIDS in protein drug or gene therapy forms. After HIV viral titer is dropped to low level by administration of drugs cocktail or vaccine, expression of the TatdMt fusions in infected cells would make the proviral HIV-1 genome virtually silent. Our strategy may prevent manifestation of the disease in short term, gradually replaces the HIV-1 infected cells with uninfected fresh cells derived from stem cells, restores immune-defense system, and ultimately may cure AIDS.

We claim:

1. The fusion proteins comprising the following elements:
 - (a) an artificial zinc-finger; and
 - (b) a TatdMt polypeptide.
2. The fusion protein of claim 1, wherein the zinc-finger recognizes human immunodeficiency virus (HIV) Long terminal repeat (LTR) region
3. The fusion protein of claim 1 further comprising POZ (Poxvirus and Zinc finger)-domain.
4. The fusion protein according to either of claims 1 or 3, further comprising a nuclear localization signal (NLS).
5. The fusion protein according to claims 1 or 2, wherein the fusion protein recognizes the HIV-1 LTR region -64 bp ~ -56 bp site and/or -67 bp ~ -59 bp site from transcription start site.
6. The fusion protein according to claims 2 or 3, wherein the fusion protein recognizes the HIV-1 LTR region -64 bp ~ -56 bp site and/or -67 bp ~ -59 bp site from transcription start site.
7. The fusion protein according to claim 1 wherein the fusion protein is HIV transcription repressor.
8. The fusion protein according to claim 3, wherein the fusion protein is HIV transcription repressor.
9. The fusion protein according to claim 1, wherein the zinc finger comprises amino acid sequence of SEQ ID NO:11 or SEQ ID NO:12.
10. The fusion protein according to claim 1, wherein the TatdMt polypeptide comprises amino acid sequence of SEQ ID NO:10.
11. The fusion protein according to claim 3, wherein the POZ-domain comprises amino acid sequence of SEQ ID NO:9.

12. A DNA molecule that encodes a fusion protein comprising a zinc finger and a TatdMt polypeptide.

5 13. A DNA molecule according to claim 12, wherein the zinc finger comprises nucleic acid sequence of SEQ ID NO:13 or SEQ ID NO:14.

14. A DNA molecule according to claim 12, wherein the TatdMt polypeptide comprises nucleic acid sequence of SEQ ID NO:15.

10 15. A DNA molecule that encodes a fusion protein comprising a POZ (Poxvirus and Zinc finger)-domain; a zinc finger; and a TatdMt polypeptide.

16. A DNA molecule according to claim 15, wherein the POZ-domain comprises nucleic acid sequence of SEQ ID NO:16.

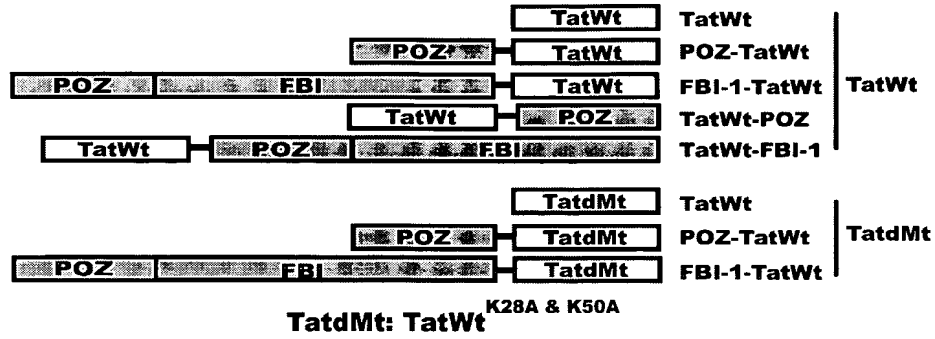
15

ABSTRACT

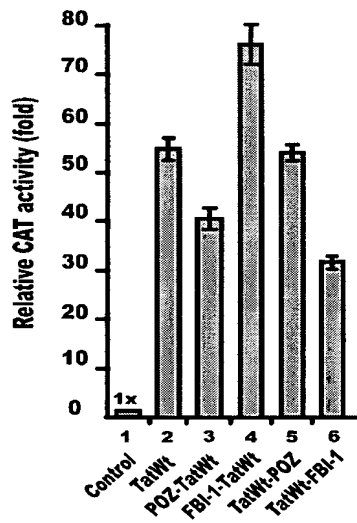
The fusion proteins are suitable for use as anti-AIDS therapeutic agents which are fused via artificial zinc fingers and the POZ-domain or artificial zinc fingers, the POZ domain and the TatdMt polypeptide.

1/8
 FIG. 1

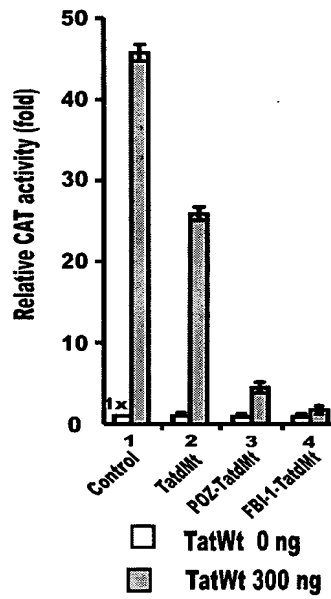
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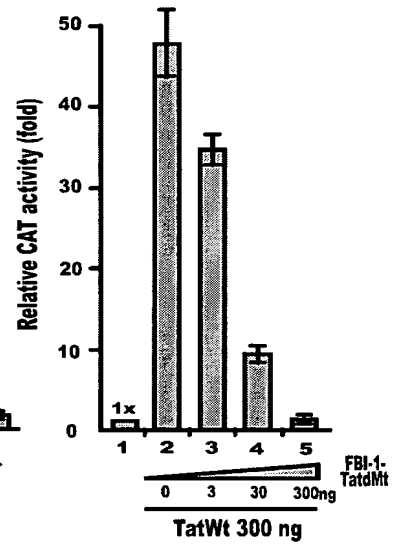
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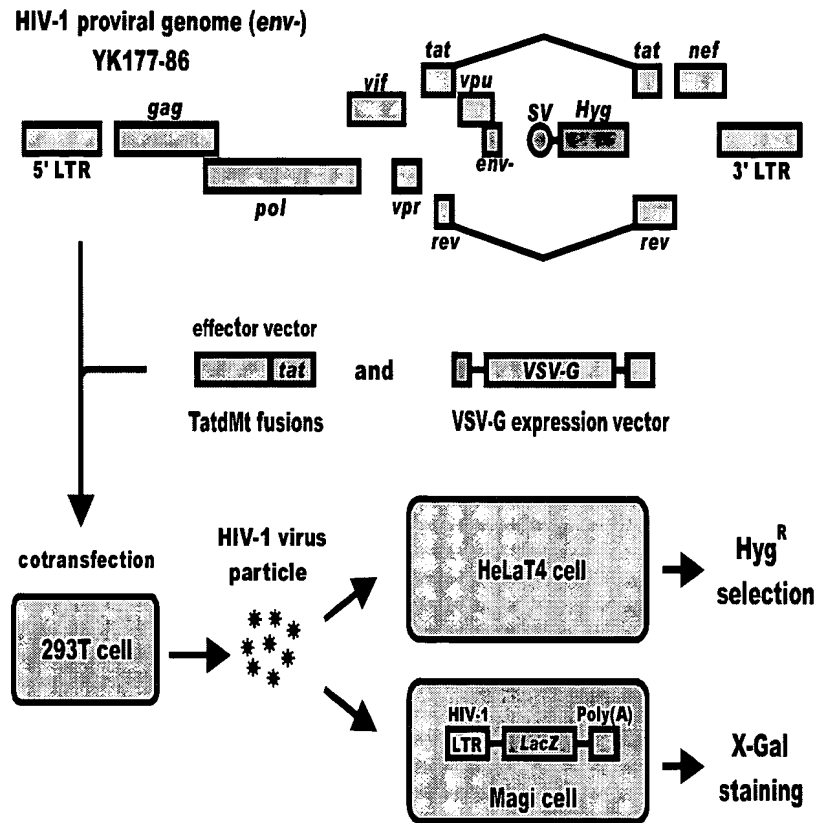
C



D



2/8
 FIG. 2

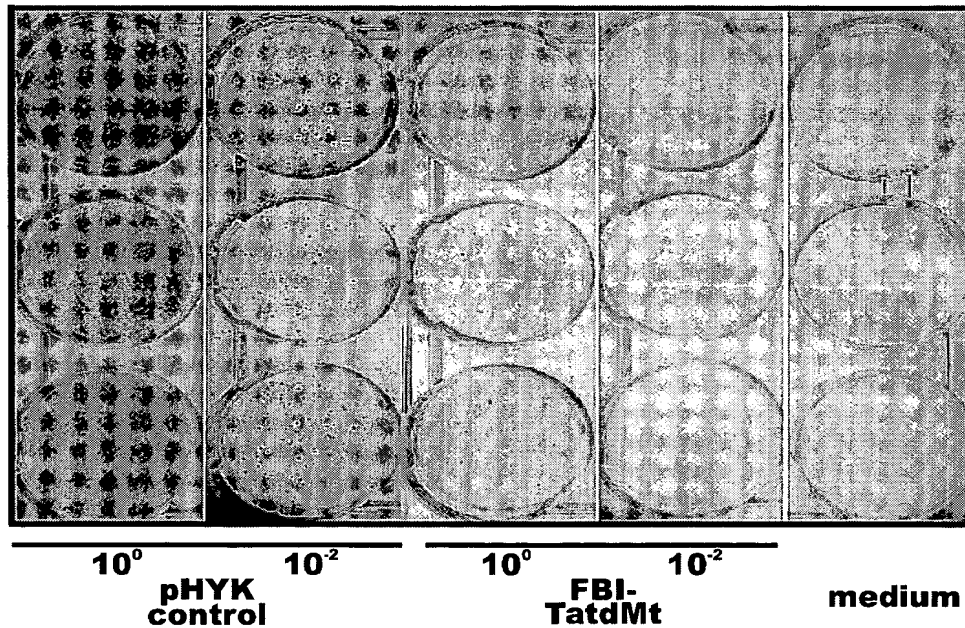


3/8
 FIG. 3

A

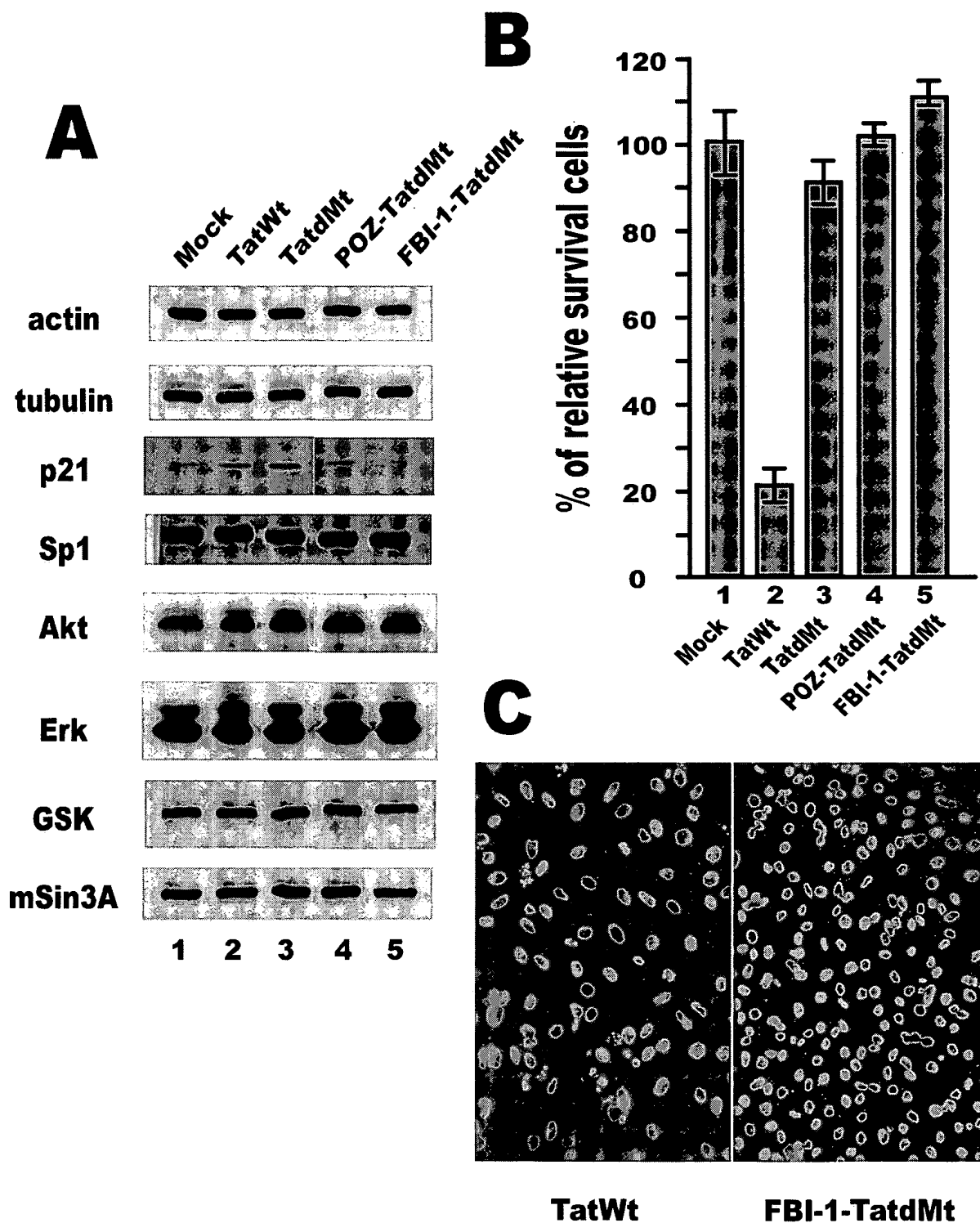
effector plasmid	colony formation assay HeLaT4 cell		β -gal assay Magi cells
	dilution rate ^a	number of Hyg ^R colonies \pm SD/ml	% of blue cells/ml ^b
PHYK (negative control)	10 ⁰	too many	+++++
	10 ⁻¹	too many	
	10 ⁻²	58 \pm 8	
FBI-TatdMt	10 ⁰	25 \pm 1	+/-
	10 ⁻¹	6 \pm 1	
	10 ⁻²	0	
control medium^c		0	-

B

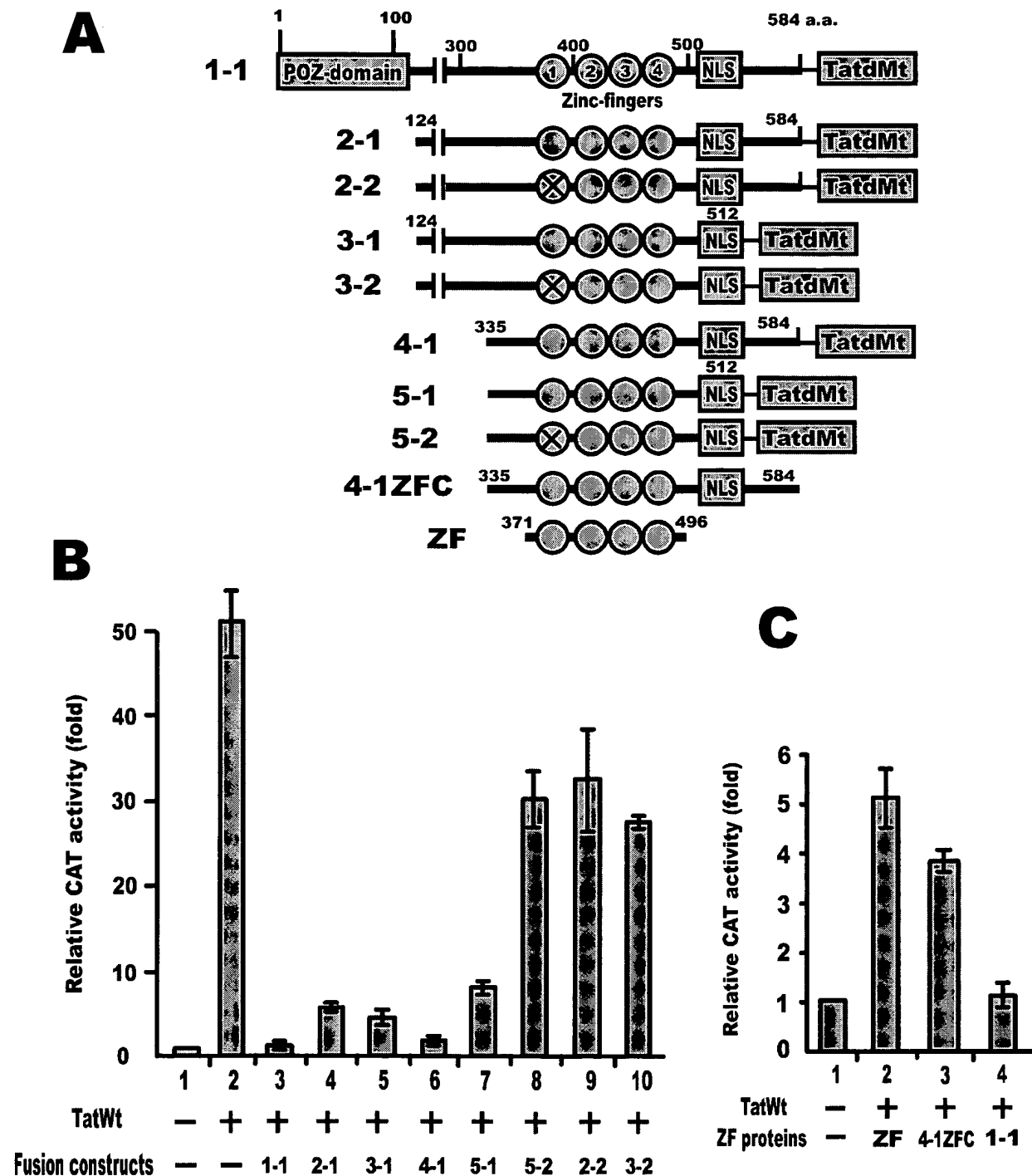


4/8

FIG. 4

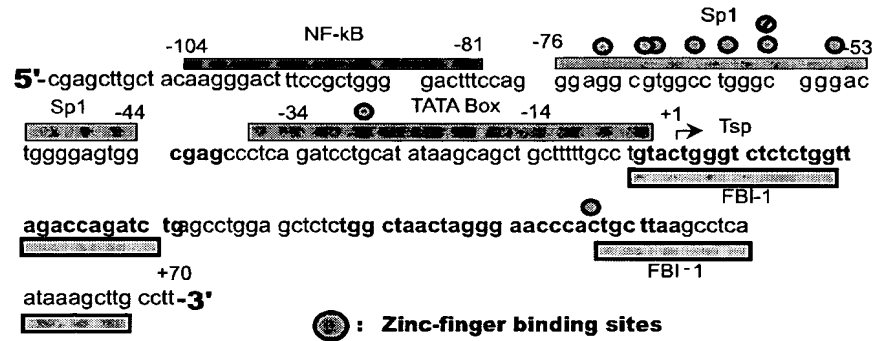


5/8
 FIG. 5

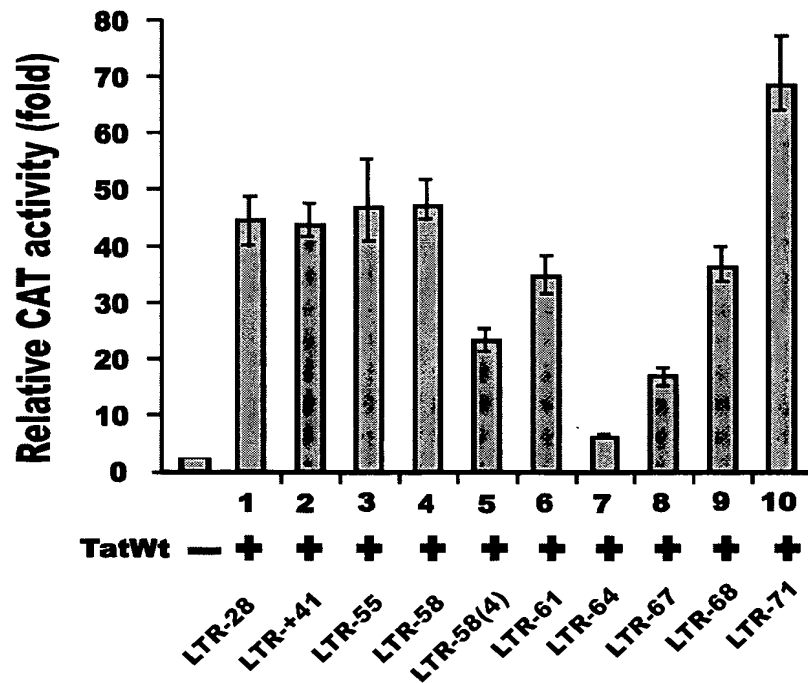


6/8
 FIG. 6

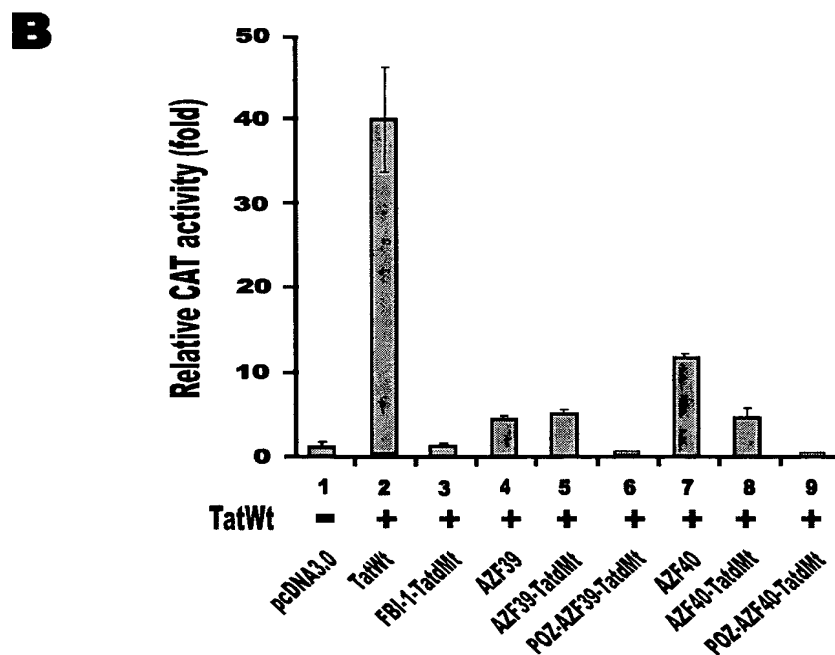
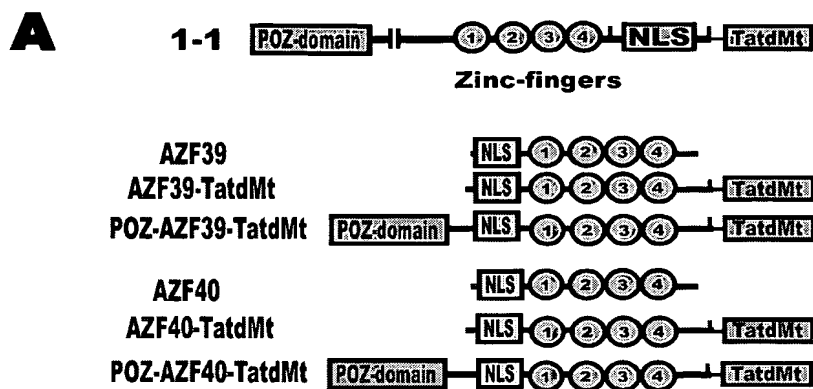
A HIV-1 LTR region



B

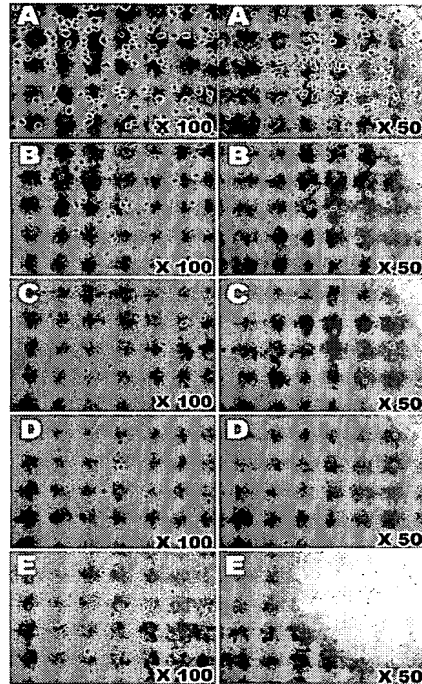


7/8
 FIG. 7



8/8
FIG. 8

A



A : pHYK

B : FBI-TatdMt

D : POZ-AZF39-TatdMt

E : POZ-AZF40-TatdMt

F : Negative Control

B

Effector plasmid	% of Blue cells/ Well
pHYK	49.7
FBI-TatdMt	12.7
POZ-AZF39-TatdMt	2.3
POZ-AZF40-TatdMt	0.6
Negative Control	0

<110> HUR, Man-Wook

<120> FUSION PROTEIN COMPRISING TATDMT POLYPEPTIDE

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<170> KopatentIn 1.71

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 20 25 30
 Leu Cys Asp Val Val Ile Leu Val Glu Gly Arg Glu Phe Pro Thr His
 35 40 45
 Arg Ser Val Leu Ala Ala Cys Ser Gln Tyr Phe Lys Lys Leu Phe Thr
 50 55 60
 Ser Gly Ala Val Val Asp Gln Gln Asn Val Tyr Glu Ile Asp Phe Val
 65 70 75 80
 Ser Ala Glu Ala Leu Thr Ala Leu Met Asp Phe Ala Tyr Thr Ala Thr
 85 90 95
 Leu Thr Val Ser Thr Ala Asn Val Gly Asp Ile Leu Ser Ala Ala Arg
 100 105 110
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 115 120 125
 Arg Gln
 130

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 20 25 30
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 50 55 60
 His Gln Val Ser Leu Ser Lys Leu Ile
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<222> (4)..(11)

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20 25 30

Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr Gly Glu Lys Pro
35 40 45

Tyr Lys Cys Lys Gln Cys Gly Lys Ala Phe Gly Cys Pro Ser Asn Leu
50 55 60

Arg Arg His Gly Arg Thr His Thr Gly Glu Lys Pro Phe Gln Cys Lys
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 35 40 45
 Tyr Lys Cys Lys Gln Cys Gly Lys Ala Phe Gly Cys Pro Ser Asn Leu
 50 55 60
 Arg Arg His Gly Arg Thr His Thr Gly Glu Lys Pro Phe Gln Cys Lys
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 85 90 95
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aggactcata ccggggaaaa accgtataaa tgtaagcaat gtgggaaggc tttggatgt 180

ccctcaaacc ttcgaaggca tggaaggact cacaccggg aaaaaccgtt ccagtgtaaa 240

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<211> 318

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aggactcata ccggggaaaa accgtataaa tgtaagcaat gtgggaaggc tttggatgt 180

ccctcaaacc ttcgaaggca tggaaggact cacaccggg aaaaaccgtt ccagtgtaaa 240

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 <223> POZ-Domain

<400> 16
 atggccggcg gcgtggacgg ccccatcggg atcccgtcc ccgaccacag cagcgacatc 60
 ctgagtgggc tgaacgagca gcggacgcag ggccgtctgt gcgacgtggt gatcctggtg 120
 gagggccgcg agttccccac gcaccgctcg gtgctggccg cctgcagcca gtacttcaag 180

aagctgttca cgtcgggagc cgtggtggac cagcagaacg tgtacgagat cgacttcgtc 240
agcgccgagg cgctcaccgc gctcatggac ttcgcctaca cggccacgct caccgtcagc 300
acagccaacg tgggtgacat cctcagcgcc gcccgctgc tggagatccc cgccgtgagc 360
cacgtgtgcg ccgacctct ggaccggcag 390

<210> 17
<211> 181
<212> PRT
<213> Artificial Sequence

<220>
<223> AZF39-TatdMt

<400> 17
Met Glu Leu Pro Pro Lys Lys Lys Arg Lys Val Gly Ile Arg Ile Pro
1 5 10 15
Gly Glu Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg
20 25 30
Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr Gly Glu Lys Pro
35 40 45
Tyr Lys Cys Lys Gln Cys Gly Lys Ala Phe Gly Cys Pro Ser Asn Leu
50 55 60
Arg Arg His Gly Arg Thr His Thr Gly Glu Lys Pro Phe Gln Cys Lys
65 70 75 80
Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr
85 90 95

Arg Thr His Thr Gly Glu Lys Ala Ala Ala Asp Ile Met Glu Pro Val
100 105 110

Asn Pro Ser Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys Thr
115 120 125

Ala Cys Thr Asn Cys Tyr Cys Ala Lys Cys Cys Phe His Cys Gln Val
130 135 140

Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Ala Lys Arg
145 150 155 160

Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser
165 170 175

Leu Ser Lys Leu Ile
180

<210> 18
<211> 181
<212> PRT
<213> Artificial Sequence

<220>
<223> AZF40-TatdMt

<400> 18
Met Glu Leu Pro Pro Lys Lys Lys Arg Lys Val Gly Ile Arg Ile Pro
1 5 10 15

Gly Glu Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg
20 25 30

Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr Gly Glu Lys Pro
35 40 45

Tyr Lys Cys Lys Gln Cys Gly Lys Ala Phe Gly Cys Pro Ser Asn Leu
50 55 60

Arg Arg His Gly Arg Thr His Thr Gly Glu Lys Pro Phe Gln Cys Lys
65 70 75 80

Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr
85 90 95

Arg Thr His Thr Gly Glu Lys Ala Ala Ala Asp Ile Met Glu Pro Val
100 105 110

Asn Pro Ser Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys Thr
115 120 125

Ala Cys Thr Asn Cys Tyr Cys Ala Lys Cys Cys Phe His Cys Gln Val
130 135 140

Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Ala Lys Arg
145 150 155 160

Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser
165 170 175

Leu Ser Lys Leu Ile
180

<210> 19

<211> 313

<212> PRT

<213> Artificial Sequence

<220>

<223> POZ-Domain-AZF39-TatdMt

<400> 19
Met Ala Gly Gly Val Asp Gly Pro Ile Gly Ile Pro Phe Pro Asp His
1 5 10 15
Ser Ser Asp Ile Leu Ser Gly Leu Asn Glu Gln Arg Thr Gln Gly Leu
20 25 30
Leu Cys Asp Val Val Ile Leu Val Glu Gly Arg Glu Phe Pro Thr His
35 40 45
Arg Ser Val Leu Ala Ala Cys Ser Gln Tyr Phe Lys Lys Leu Phe Thr
50 55 60
Ser Gly Ala Val Val Asp Gln Gln Asn Val Tyr Glu Ile Asp Phe Val
65 70 75 80
Ser Ala Glu Ala Leu Thr Ala Leu Met Asp Phe Ala Tyr Thr Ala Thr
85 90 95
Leu Thr Val Ser Thr Ala Asn Val Gly Asp Ile Leu Ser Ala Ala Arg
100 105 110
Leu Leu Glu Ile Pro Ala Val Ser His Val Cys Ala Asp Leu Leu Asp
115 120 125
Arg Gln Gly Tyr Met Glu Leu Pro Pro Lys Lys Lys Arg Lys Val Gly
130 135 140
Ile Arg Ile Pro Gly Glu Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg
145 150 155 160
Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr
165 170 175
Gly Glu Lys Pro Tyr Lys Cys Lys Gln Cys Gly Lys Ala Phe Gly Cys
180 185 190

Pro Ser Asn Leu Arg Arg His Gly Arg Thr His Thr Gly Glu Lys Pro
195 200 205

Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu
210 215 220

Lys Thr His Thr Arg Thr His Thr Gly Glu Lys Ala Ala Ala Asp Ile
225 230 235 240

Met Glu Pro Val Asn Pro Ser Leu Glu Pro Trp Lys His Pro Gly Ser
245 250 255

Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Ala Lys Cys Cys Phe
260 265 270

His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly
275 280 285

Arg Ala Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
290 295 300

His Gln Val Ser Leu Ser Lys Leu Ile
305 310

<210> 20

<211> 313

<212> PRT

<213> Artificial Sequence

<220>

<223> POZ-Domain-AZF40-TatdMt

<400> 20

Met Ala Gly Gly Val Asp Gly Pro Ile Gly Ile Pro Phe Pro Asp His

1	5	10	15
Ser Ser Asp Ile Leu Ser Gly Leu Asn Glu Gln Arg Thr Gln Gly Leu			
20	25	30	
Leu Cys Asp Val Val Ile Leu Val Glu Gly Arg Glu Phe Pro Thr His			
35	40	45	
Arg Ser Val Leu Ala Ala Cys Ser Gln Tyr Phe Lys Lys Leu Phe Thr			
50	55	60	
Ser Gly Ala Val Val Asp Gln Gln Asn Val Tyr Glu Ile Asp Phe Val			
65	70	75	80
Ser Ala Glu Ala Leu Thr Ala Leu Met Asp Phe Ala Tyr Thr Ala Thr			
85	90	95	
Leu Thr Val Ser Thr Ala Asn Val Gly Asp Ile Leu Ser Ala Ala Arg			
100	105	110	
Leu Leu Glu Ile Pro Ala Val Ser His Val Cys Ala Asp Leu Leu Asp			
115	120	125	
Arg Gln Gly Thr Met Glu Leu Pro Pro Lys Lys Lys Arg Lys Val Gly			
130	135	140	
Ile Arg Ile Pro Gly Glu Lys Pro Phe Gln Cys Lys Thr Cys Gln Arg			
145	150	155	160
Lys Phe Ser Arg Ser Asp His Leu Lys Thr His Thr Arg Thr His Thr			
165	170	175	
Gly Glu Lys Pro Tyr Lys Cys Lys Gln Cys Gly Lys Ala Phe Gly Cys			
180	185	190	
Pro Ser Asn Leu Arg Arg His Gly Arg Thr His Thr Gly Glu Lys Pro			
195	200	205	

Phe Gln Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu
 210 215 220

Lys Thr His Thr Arg Thr His Thr Gly Glu Lys Ala Ala Ala Asp Ile
 225 230 235 240

Met Glu Pro Val Asn Pro Ser Leu Glu Pro Trp Lys His Pro Gly Ser
 245 250 255

Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Ala Lys Cys Cys Phe
 260 265 270

His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly
 275 280 285

Arg Ala Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
 290 295 300

His Gln Val Ser Leu Ser Lys Leu Ile
 305 310

<210> 21

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Forward primer for pcDNA3.0-artificial zinc-finger fusion TatdMt
 constructs

<400> 21

gatcgggtacc atggaattgc ctcaaaaaa gaag

34

<210> 22

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Reverse primer for pcDNA3.0-artificial zinc-finger fusion TatdMt constructs

<400> 22

gatcgatc tgccgccgct tttcaccgg tatg

34